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RICKETTSIAL DISEASES: SCRUB TYPHUS AND
TRENCH FEVER

Susumu Ito, Ph.D.

FINAL PROGRESS REPORT

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tsutsugamushi in BHK₂₁ cells as a monolayer or in suspension cultures. Irradiated cells seem to enhance rapid rickettsial proliferation. Solubilization after freezing and thawing and by sonication and Triton X-11 treatment was used to isolate soluble antigen for characterization and further purification.

An enzyme immunoassay test to detect antibodies to Rochalimaea quintana in sera from patients with trench fever was evaluated with soluble antigens. This test detected antibodies in sera from known cases that were negative to other serological tests. However, there is an antigen that cross reacts with other rickettsia which appears to be a lipopolysaccharide antigen. This lipopolysaccharide seems to be related serologically to scrub typhus antigen.

In these studies, infected cells as well as isolated samples used in the studies were fixed and examined by electron microscopy to monitor the structure of the organism and the host cell as well as some of the isolated and purified samples. Studies undertaken towards the end of this support and subsequently completed or in progress include several attempts to characterize the solubilized antigen extracted from R. tsutsugamushi. An extensive study on the phagocytosis and release of rickettsiae by polymorphonuclear leukocytes has been completed and a study on the immunoferritin labeling of scrub typhus rickettsiae has also been completed.

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Foreward

This Final Report covers studies undertaken on rickettsial diseases and supported by the US Army Medical Research and Development Command. These undertakings are a continuation of an ongoing project which has been directed towards the isolation and purification of rickettsial antigens and the detection of rickettsial antibodies of scrub typhus and trench fever.

In raising antibodies, withdrawing antisera, and performing toxicity tests the investigators adhered to the "Guide for Laboratory Animal Facilities and Care" as promulgated by the Committee on the Guide for Laboratory Animal, Resources, National Academy of Sciences-National Research Council.

Summary

This study was directed towards the isolation of rickettsial antigens and detection of antibodies. Stock supplies of Gilliam, Kato and Karp strains of Rickettsia tsutsugamushi were propagated in embryonated chick eggs and used for infecting cultured cells. Infected McCoy cell extracts containing soluble antigen as well as infected yolk sacs were inoculated into guinea pigs to produce antisera. However, the yield of antibody was low and only detectable by fluorescent antibody techniques. More recently progress was made in growing R. tsutsugamushi in BHK₂₁ cells as a monolayer or in suspension cultures. Irradiated cells seem to enhance rapid rickettsial proliferation. Solubilization after freezing and thawing and by sonication and Triton X-100 treatment was used to isolate soluble antigen for characterization and further purification.

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Personnel: The changes which have occurred during the final grant have been marked and most of these have been detailed in the various letters and reports that we have made to this granting agency. Since July 1, 1976 Dr. J. William Vinson has been retired from active academic duties in the Department of Microbiology. However, he was still retained and on salary until January 1, 1977. He still frequently visited the laboratory and helped us by advising us on many aspects of rickettsial cultivation and related serological studies. During the coming year, he has agreed to continue his interest in our work and will collaborate with us in some aspects of the proposed studies.

Dr. Jack Whitescarver who shared the major daily responsibility of managing his program in rickettsial studies has resigned his research associate position to take on an administrative post at NIH on June 20, 1977. His loss to the program put the present study in a precarious position but fortunately, Dr. A. Bruce MacDonald who was already a Professional Assistant on this Research Contract became available and interested in taking on a major responsibility in this project. In the relatively short period of just over two months, Dr. MacDonald and his research assistant have made rather remarkable strides in the cultivation as well as purification of antigens from Rickettsia tsutsugamushi. Some of these early results are elaborated in various parts of this proposal.

More recently we have added Drs. Y. Rikihisa and C.M. Ho to continue our studies on R. tsutsugamushi. Ms. T. Rota continued as a research assistant and Mr. L. Tun Ho a graduate student did the antigen isolation and purification studies.

Since July of 1978, Dr. MacDonald took the chairmanship of the Microbiology Department at University of Massachusetts in Amherst, MA and Ms. T. Rota transferred to a position at the Mass General Hospital. During the past year rickettsial studies have been carried out by Drs. Rikihisa, Ho and Ito.

RESEARCH PROGRESS: During the course of this grant period a number of unforeseen changes in personnel in the professional and research assistant group has taken place. In spite of these events it is felt that we have made considerable progress on this contract.

Dr. Michael Hollingdale completed his program of work on the antigens of Rickettsia quintana. An enzyme immunoassay (ELISA) test for detecting antibodies to R. quintana in sera from patients with trench fever and further evaluated, using the soluble antigen previously described. The test proved highly sensitive and detected antibodies in several sera from known trench fever cases that were negative in other serological tests. However, some sera from other rickettsial infections also reacted. Nine of 9 scrub typhus sera were positive, as were several epidemic and endemic typhus sera, and a single Rocky Mountain spotted fever serum. The cross-reacting antigen appeared not to be cytoplasmic protein but a cell envelope lipopolysaccharide (LPS) antigen. This confirms the results of counterimmunoelectrophoresis (CIE) where cross reaction using solubilized cell envelope antigens occurred but no cross reactions occurred with soluble antigen. It is likely that the absence of cross reactions of the soluble antigen in CIE compared to ELISA is due to the lower sensitivity of the CIE test and the small amounts of LPS in the soluble antigen released by sonication (as reported earlier) that are reactive only in ELISA.

During this program, LPS has been demonstrated in R. quintana by various procedures; It is the first observation of rickettsial LPS. It appears serologically related to a similar component in R. tsutsugamushi. The possibility exists in addition that rickettsial LPS is similar, if not identical, in all species. Numerous serological tests have been described, including passive hemagglutination, latex agglutination, radioimmunoprecipitation, CIE and ELISA. Each test differs in sensitivity, specificity and ease of operation, with CIE being the most suitable for day-to-day use, particularly for large numbers of sera. Other antigens of R. quintana have been investigated and described. It is felt that these results are of considerable use and application to the other rickettsial species and for the elucidation of rickettsial pathogenicity. The owl monkey experiments with Rochalimaea quintana were continued. At two-weekly intervals, the infected monkeys were bled and the blood screened for rickettsemia and serum antibody.

Stock supplies of Gilliam, Kato and Karp strains of scrub typhus were made by serial propagation in six day embryonated chicken eggs. Freshly harvested scrub typhus infected yolk sac was homogenized and used at a 10% concentration to infect cell cultures. Vero, BT20, tick, and McCoy cells were infected and all cell lines supported rickettsial growth. However, McCoy cells provided the best results with a 4+ infection in 3 days. This cell system was used to propagate scrub typhus for attempts at soluble antigen preparation.

Guinea pigs were used to raise antisera against the yolk sac grown scrub typhus. The antigen was prepared by solubilizing with sonication followed by centrifugation. The soluble antigen was inoculated in guinea pig foot pads with Freund's complete adjuvant. The antisera acquired by this method was of such a low titer that no precipitin bands could be observed on immunodiffusion plates. However, fluorescent antibody (FA) techniques indicated a low level of antibody directed toward the scrub typhus rickettsia. After consulting with Ms. Marilyn Bozeman, we inoculated guinea pigs intra-cerebrally with the yolk sac grown antigen and collected antisera in 28 days.

To eliminate precipitin bands associated with yolk sac components, soluble antigen was prepared from scrub typhus agent grown in McCoy cells. The host cell was lysed by freezing and thawing; the resultant material was sonicated in the presence of 1% v/v Triton X-100 and partially clarified by centrifugation. The supernatant was concentrated 3-fold by dialysis against ethylene glycol. The final soluble antigen provided no detectable precipitin lines against the 28 day antisera. However, FA demonstrated antibody directed to scrub rickettsia even though at low titers. The soluble antigen was placed on an acrylamide gel electrophoretic system along with solubilized McCoy cells for a control. Only parallel bands were detected, indicating our rickettsial antigen is at a very low level. Therefore, we are now working at increasing the concentration of rickettsial antigen. Our first approach is to double the mount of gradient centrifugation according to Obijeski and Palmer (personal communication) using potassium tartrate and glycerol.

Throughout this period we have made routine fixations for electron microscopy of scrub typhus microorganisms grown in the McCoy cells and in some of the chick yolk sacs from infected embryos. Thin sections of

embedded material were examined to confirm the Giemsa stained smears for the frequency and intracellular localization of the rickettsia. A series of electron micrographs of R. tsutsugamushi in infected cells has been made.

We have found the R. tsutsugamushi grows well in BHK₂₁ cells whether grown as a monolayer or in suspension cultures. The previous work on McCoy cells where these same rickettsiae could be grown in great abundance was discontinued because of the presence of numerous mycoplasma and C type virus particles normally present in this cell line. These were observed by electron microscopy.

In an attempt to induce rapid proliferation and larger numbers of rickettsiae, BHK₂₁ cells were irradiated with 5,000 R from a cobalt source prior to inoculation with R. tsutsugamushi. This radiation inhibits cell division and proliferation of the BHK₂₁ cells but does not kill them. Furthermore, this treatment does not seem to cause marked ultrastructural changes in the cultured cells but irregular shaped nuclei and multinucleated cells were common. In these irradiated cells the rickettsia grew in great abundance within days compared to the weeks required for a lower concentration of rickettsiae.

The recent studies have been directed towards the phagocytosis and entry of R. tsutsugamushi into macrophages. To elucidate the ultrastructural features of rickettsial interactions with host cells, Rickettsia tsutsugamushi (Gilliam strain) were serially propagated in monolayers of BHK₂₁ cells and incubated in vitro with guinea pig peritoneal polymorphonuclear leukocytes obtained after casein-Na injections. Suspensions of heavily infected BHK cells or highly enriched pellets of extracellular R. tsutsugamushi were incubated with the polymorphonuclear leukocytes for periods up to 4 hrs at 35°C and fixed for electron microscopy. Within 30 min some of these phagocytic leukocytes had ingested significant numbers of rickettsiae. When infected BHK cells were used as the source of rickettsiae about half of the internalized microorganisms were still sequestered in phagosomes but the remainder had escaped from the phagosomes and resided in the host cell cytoplasm. In contrast, when suspensions of host cell-free extracellular rickettsiae were used under identical incubation conditions, about 90% of the phagocytosed rickettsiae were still confined within phagosomes and the remainder were free in the leukocyte cytoplasm.

The process of rickettsial escape from the phagosome into the glycogen-packed areas of the cytoplasm of the polymorphonuclear leukocyte was followed by observations on what appeared to be various stages of the process. Many of the Rickettsiae confined to phagosomes were in various stages of autolysis. Examples of rickettsiae in phagosomes with ruptured membranes with phagosomal space containing glycogen granules were observed. With continued incubation larger members of rickettsiae were found in the phagosomes as well as in the cytoplasm.

Various electron dense tracers were used to follow the process of rickettsial uptake by the phagocytes. When thorium dioxide was used it labeled the outer surface of the polymorphonuclear leukocyte and lined the phagosomal membrane surrounding the rickettsiae. When such rickettsiae escaped from the phagosome, bits of thorium dioxide labeled membrane fragments were present in the cytoplasm. When ferritin was used as extracellular marker, it appeared to cause the leukocyte to phagocytize rickettsiae while it was still enclosed within an envelope of the BHK cell plasma membrane. Very little BHK cell cytoplasm was usually apparent but in some instances moderate

amounts of the initial host cell cytoplasm was included in the phagosome. Heat treated rickettsiae (56°C for 5 min) were phagocytosed extensively as a mass and resided in glycogen-rich pools. However, after longer heat treatment (59°C for 20 min) rickettsiae remained in phagosomes and did not enter the cytoplasm. Formaldehyde or glutaraldehyde fixation decreased phagocytosis and inhibited the release of rickettsiae from phagosome. Cytochalasin B completely inhibited phagocytosis of rickettsiae by leukocytes. This study indicates that the uptake of viable rickettsiae into leukocytes occurs by phagocytosis and subsequent breakage of the phagosomal membrane.

A study which has been completed is the following on immunolabeling with ferritin. The immunolabeling characteristics of Rickettsia tsutsugamushi (Gilliam strain) were examined by employing an indirect immunoferritin technique for electron microscopy. Purified IgG fraction of antibody to R. tsutsugamushi raised in rabbits was reacted with formalin fixed rickettsiae and then complexed with ferritin conjugated goat anti-rabbit antibody. R. tsutsugamushi which were cultivated in yolk sacs were used to raise antibody and for determining their immunolabeling characteristics. When rickettsiae in BHK cells infected from yolk sac seed material were reacted for the immunoferritin labeling, the binding of ferritin was found to be dense and uniform on the outer surface of the rickettsiae in disrupted BHK cells. Rickettsiae within intact host cells were not labeled and host cell components were also not labeled. A number of small 100m μ vesicles were labeled with ferritin-labeled antibody and interpreted to be vesiculated remnants of rickettsiae resulted in the uniform ferritin labeling of the microorganism. Varying degrees of aggregation of rickettsiae which appeared to depend upon the purity of the pellets was observed. The labeling of R. tsutsugamushi by the indirect immunoferritin technique examined at high magnification reveals the ferritin label very close to the outer dense leaflet of the cell wall. On some rickettsiae or on focal sites of others, the ferritin label may be of several ferritin particles in depth suggesting the presence of a coating.

When the immunoferritin labeling characteristics of R. tsutsugamushi were examined during successive serial passages in BHK cells, it was found that the frequency of labeling decreased with each passage. By the 10th passage, there was no detectable labeling. When these rickettsiae were inoculated back into chicken yolk sacs, they regained their immunoferritin labeling characteristics. Rickettsiae from the 20th serial passage in BHK cells required 4 passages in yolk sacs to regain their initial labeling characteristics while R. tsutsugamushi passed back into yolk sacs after the fourth serial propagation in BHK cells regained their labeling affinity on the first passage of yolk sacs. Rickettsiae which did not label (after 30 passages in BHK cells) regained some of their labeling characteristics when sonicated or when cultivated in the presence of guinea pig polymorphonuclear leukocytes.

The immunolabeling characteristics of R. tsutsugamushi and changes under different conditions will be illustrated.

Isolation of rickettsial antigens.

Extracellular pellets of R. tsutsugamushi obtained after serial passages in BHK₂₁ cells were suspended in 0.1 M borate buffered saline, pH 8.5 and cooled to 0°C. One μ m Cl of ¹²⁵Iodine was used to label the

rickettsiae by the Bolton-Hunter technique. After incubation in the ^{125}I iodinated p-hydroxyphenylpropionic acid, N-hydroxysuccinimide ester for 1 hour, the mixture was solubilized in Nonidet P-40 (0.05% final concentration) and incubated at 37°C . for 1 hour. The suspension was centrifuged for twenty minutes at $14,000\times g$ at 4°C . The supernatant fluid was harvested and subjected to gel filtration on Sepharose 4B. The elution profile is shown in Figure 1. Three main peaks are evident as monitored by counting ^{125}I disintegrations. As shown in Table 1 approximately 25% of the radioactivity from fractions B and C of pool peak 2 (figure 1) were specifically bound by antisera prepared in a rabbit against R. tsutsugamushi. The antigen used was prepared in BHK $_{21}$ cells and treated with formalin prior to injection.

Although the control antisera prepared against whole BHK $_{21}$ cells bound more radioactivity than the pre-immune sera from the rabbit inoculated with BHK $_{21}$ cell cultivated rickettsiae, the difference between the amount of radioactivity bound by the infected cell preparations was significantly higher. Fractions A and D contained less overall antigenic activity. We are currently subjecting all the peak fractions from this experiment to further analysis. Initial ion exchange chromatography of the second peak (A,B,C,D) has resulted in a nearly complete loss of detectable activity. The factors responsible for this loss are being investigated.

Related publications of studies supported in part from this contract.

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In Press:

Ito, S. and Vinson, J.W. *Rickettsia* in flea gut tissues. Publication of the International Conference on Fleas. ed. by Miriam Rothschild, 1979.

Papers in Preparation:

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Rota, T.R., Lee, T.H., Ito, S., Rikihisa, Y., and MacDonald, A.B. Isolation of purified pellets of *Rickettsia tsutsugamushi* from serially infected cell cultures (abstract presented at the 1978 Annual Meeting of the American Society for Microbiology, Las Vegas, Nevada).

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Staffing:

Susumu Ito, Professor of Anatomy, June 1973 to present
J. William Vinson, Associate Professor of Microbiology, June 1973 to July 1976
Jack Whitescarver, Postdoctoral Fellow, June 1975 to June 1977
A. Bruce MacDonald, Associate Professor of Microbiology, July 1972 to October 1978
Yasuko Rikihisa, Research Fellow, October 1977 to present
Chau-Mei Ho, Research Fellow, January 1977 to present

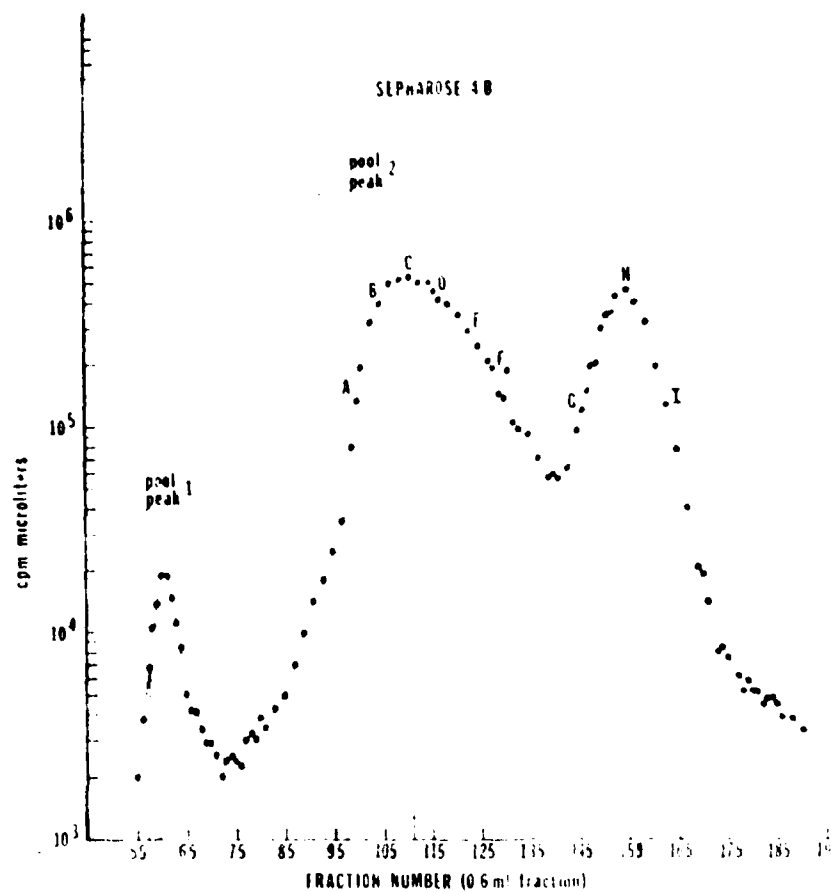


FIG. 1.

Gel filtration of *R. tsutsugamushi* extract (see text) on Sepharose 4B

Table I
Radioimmunoprecipitation of *Rickettsia Tsutsugamushi* Fractions

Sample (4) Fr. ction	Counts Per Minutes Precipitated				
	Total Counts per min. (input)	Immune Serum	Control Serum	% precipitated	Ratio Immune/ control
A	119,590	26,030	3,569	21.7	7.3
B	419,230	101,172	7,217	24.1	14.0
C	497,112	107,330	10,905	21.6	9.8
D	450,060	38,023	10,591	8.4	3.6

(4) Fractions of pool 2 (Figure 1)

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